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Supplementation of two novel probiotics in the diet of lactating dairy cows

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The student author and the program of study committee are solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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ABSTRACT

Forty-eight multiparous Holstein cows (121 ± 22 DIM) were used in a randomized complete block design to evaluate supplementation of two probiotic strains *Pediococcus acidilactici 19839* (**PED**) or *Bacillus subtilis* 15541 (**BAC**) on the effect of milk yield and composition, total tract nutrient digestibility, rumen pH and volatile fatty acid concentration. Cows were housed in a free-stall barn, milked three times a day, and fed twice daily for 105 d. All treatments consisted of a basal TMR diet, top-dressed with a specific supplement: 1) control (**CON**) with no probiotics; 2) **PED** fed at 1×10^{10} CFU/d; 3) **BAC** fed at 1×10^{10} CFU/d and; 4) basal TMR supplemented with a combination of *Enterococcus faecium at* 1×10^{10} CFU/d and yeast (**PRO**). Individual feed intake and milk yield data were recorded daily and averaged weekly. Two blocks contained rumen fistulated cows and were used for rumen pH measurements and rumen fluid collection on d 105. Data were analyzed using a mixed model with week, treatment and their interaction as fixed effects with pre-experiment milk yield as a covariate and cow and block as random effects. Dry matter intake was similar across treatments with an average of 24.3 ± 0.8 kg/d. Milk yield averaged 37.4 ± 1.4 kg/d across treatments; analyses of data in 5-wk periods showed that **PED** resulted in additional 3.9 ± 2.9 kg/d for the first period compared to all other treatments. Concentration of milk fat and protein were similar across treatments with averages of 3.63 ± 0.02 % and 3.05 ± 0.06 %. Digestibility of dry matter, organic matter, and protein were similar across treatments and averaged 66.65 ± 1.48 %, 68.88 ± 1.43 %, and 67.11 ± 1.81 %. Similarly, acid and neutral detergent fiber digestibility was similar for all treatments. Mean daily

rumen pH was 5.69 ± 0.05 across treatments. Proportions of acetate, propionate and butyrate averaged 57.1 ± 1.8%, 26.6 ± 2.3% and 11.1 ± 0.7%, across treatments respectively. Although the mechanism for transient increase in milk yield remains to be elucidated, the results demonstrate that, in dairy cattle, supplementation with the specific strain *P. acidilactici* 19839 has the potential to improve lactation performance without detrimental effects on digestibility, rumen pH, and VFA concentration.

CHAPTER 1. INTRODUCTION

Antibiotics, including ionophores, have been fed to livestock to improve growth rates, feed efficiency, and increase milk production (McDougall et al., 2004, Gallardo et al., 2005). Feeding additives containing antimicrobial agents may have direct or indirect effects on animals and human health (Landers et al., 2012). Even though it is still unclear if the addition of growth promoting antimicrobial agents in animal feeding is an underlying problem for antimicrobial resistance, consumer's preferences pressure producers to search for alternative additives. Probiotics, also known as direct-fed microbials (DFM), are one alternative (Jouany and Morgavi, 2007). Since probiotics are deemed safe and already used in a wide variety of food products, consumers are more accepting with their use (Fuller, 1989). In addition to DFM, there are other substrates called prebiotics; these facilitate specific adaptations in composition and activity of the gut microflora that can convey a positive effect on the health of the host (Slavin, 2013).

The definition of probiotics has evolved over time; originally, probiotics were said to be live microorganisms that promote growth of another (Lilly and Stillwell, 1965). Later, Parker (1974) described them as a feed supplemented to animals that has a beneficial effect on the gut flora. Currently, the World Health Organization (WHO, 2002) defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host".

Some positive health benefits of probiotics in humans and small rodents are broadly stated by Schrezenmeir and de Vrese (2001) as lower frequency and duration of diarrhea, stimulation of humoral and cellular immunity, and decrease in unfavorable metabolites. Chaucheyras-Durand and Durand (2010) and Jouany and Morgavi (2007) describe potential benefits of DFM in ruminants, including a decrease in methane production, reduction of feed protein degradation, reduction in rapid fermentation of carbohydrates and modulation of lactic acid concentration, and improvements in fiber digestibility. Even though feeding sub-therapeutic dosing of antibiotics helped with most of the aforementioned benefits of DFM, the European Union has placed a ban on feeding antibiotics to livestock (EU regulation no. 1831/2003 of the European Parliament and of the Council of 22 September 2003). Therefore, probiotics are an appealing alternative because they have the potential to elicit positive effects on animal performance and public acceptance is favorable.

It is important to highlight that probiotics are live microorganisms and that their beneficial effects may be affected by a myriad of conditions such as species, host microbial species, and diet. Influencing factors that determine the effects of probiotics have been described by Seo et al. (2010) and include dosage, timing, specific strain of probiotic, and animal conditions. If the purpose of the probiotic is to target the rumen, it must be active and remain viable in such environment. Because of these requirements, the research is limited to a few genera such as *Enterococcus, Lactobacillus, Bifidobacterium, Streptococcus, Bacillus,* and *Propionibacterium*. These bacteria are most commonly used as DFM for ruminants and are classified as lactic acid-

producing (**LAB**), lactic acid-utilizing (**LUB**), or other. In addition to those, yeast DFM commonly containing *Saccharomyces cerevisiae* are supplemented in ruminal diets.

Different probiotics affect the gastrointestinal tract through diverse pathways. Lactic acid-producing bacteria have four common modes of action in ruminants: constant lactic acid supply, adaptation to the lactic acid accumulation, stimulation of lactate utilizing bacteria, and stabilization of pH (Seo et al., 2010). Lactic acid-utilizing bacteria have five modes of action that include: conversion of lactate to volatile fatty acids (**VFA**), production of propionic acid, decrease methane production, increase feed efficiency, and increase rumen pH. Yeast DFM have six different modes of action: reduction of ruminal oxygen, inhibition of excess lactic acid, supplying organic acids and vitamin B growth factors, increase microbial activity and numbers in rumen, and increase ruminal end products.

Direct-fed Microbials for Ruminants

Effects of Direct-fed Microbials on Rumen Metabolism

Effects on Acidosis and pH Regulation

Adult ruminants are fed DFM during periods of stress; for example, switching from a mostly forage-based to a high-concentrate diet (Jouany and Morgavi, 2007) involves drastic changes in substrate availability and rumen fermentation profile. Concentrates are rapidly fermented in the rumen leading to a sudden increase in the concentration of VFA with a concomitant reduction in rumen pH. Lactate has a lower pKa and it further contributes to the decrease in pH of the rumen. Fiber digesting bacteria are inhibited at lower pH, which leads to a decrease in fiber digestibility. In

addition, acidosis also inhibits the bacteria responsible for biohydrogenation of unsaturated fatty acids (Oetzel, 2007), therefore more *trans* fatty acids escape the rumen and are absorbed in the small intestine.

Feeding long-stem forages to cows stimulates mastication; an increase in amount of time spent chewing promotes saliva production, which acts as a natural buffer to maintain pH in the rumen. Since grains have smaller particles, they promote less mastication, thus reducing salivary secretion which in turn reduces buffering capacity in the rumen (Maekawa et al., 2002). Rumen acidosis occurs when animals lose buffering capacity in the rumen due to rapid accumulation of VFA, a reduction in salivary secretion or a combination of both factors. The onset of subacute rumen acidosis (**SARA**) is considered when the pH in the rumen falls below 5.8 (Kleen et al., 2003). High producing dairy cows that consume a total mixed ration (**TMR**) can be prone to SARA when the feed particles may not be long enough to stimulate mastication and the inclusion of readily fermentable carbohydrates can lead to a faster rate of fermentation and accumulation of VFA.

The rationale for feeding LAB such as *Enterococcus* and *Lactobacillus* is that the activity of these bacteria could create a low steady concentration of lactate in the rumen, thus providing a constant stimulation of LUB to prevent accumulation of lactate and reduce the risk for acidosis (Nocek et al., 2002). Alternative methods suggest the inclusion of *Megasphaera elsdenii,* a LUB, to prevent sudden drops in rumen pH from lactate accumulation (Kung and Hession, 1995). In addition, *Propionibacteria* may improve energetics in dairy cows because this type of bacteria ferment lactate to

propionate, which is the precursor for glucose in dairy cows (Reynolds et al., 2003). In addition, these bacteria can also decrease the amount of hydrogen available for methane production (Stein et al., 2006). Yeast frequently increase bacterial numbers in the rumen (Seo et al., 2010), in addition, yeast can compete with starch utilizing bacteria for fermentation (Lynch and Martin, 2002) preventing lactate build up (Chaucheyras et al., 1996). Because of their pH regulation and oxygen scavenging actions, yeast create better conditions for cellulolytic activity by bacteria (Roger et al., 1990), leading to increased forage utilization.

Effects on Volatile Fatty Acid

Different VFA concentrations and ammonia have been observed in several studies feeding DFM to dairy cows. Propionate is produced via two main pathways in the rumen: succinate pathway or the acrylate pathway (Louis et al., 2014). The succinate pathway is used when fermenting carbohydrates, lactate or succinate to produce propionate. The acrylate pathway uses lactate and acrylate analogues to produce propionate. One other pathway is the propanediol pathway used for deoxyribose sugars, however it is not as common.

Stein et al. (2006), Weiss et al. (2008), and Peng et al. (2012) observed increases in propionate concentrations when feeding DFM with either *Propionibacterium* or *Bacillus subtilis natto*. However, Raeth-Knight et al. (2007) did not report any differences in ammonia or total VFA concentration. Sun et al. (2012) reported that total VFA concentrations increased with inclusion of *Bacillus subtilis* natto. In this same study, they reported an increase in molar proportions of propionate and valerate compared to the pre-trial levels. Since propionate is the precursor for gluconeogenesis, an increase in the concentration of this VFA can mean more glucose could be available for milk production. Qiao et al. (2010) also reported an increase in total VFA concentration, but instead concentrations of acetate were greater with supplementation of *Bacillus licheniformis.* Chiquette et al. (2008) fed *Prevotella bryantii* (25A) and reported higher acetate and butyrate concentrations, interestingly, these cows had higher milk fat percentage.

Effects of Direct-fed Microbials on the Lower Gastrointestinal Tract

Modes of action for probiotics further in the gastrointestinal tract (GIT) include antimicrobial compound production, competing for colonization and for nutrients, enzyme production or stimulation, immune response, metabolizing and detoxifying undesirable compounds (Seo et al., 2010). Studies have established probiotics effectiveness in the lower GIT; Lee et al. (2003) noted that supplementing *Lactobacillus* to humans limited pathogens from attaching to receptors in the intestinal epithelial cells. Matsuguchi et al. (2003) reports various LAB can stimulate an immune response by activating macrophages.

Feeding Direct-fed Microbials to Dairy Cows

Effects of Direct-fed Microbials on Lactation Performance

Direct-fed microbials fed to lactating dairy cows have been reported to increase DMI (Nocek et al., 2003; Nocek and Kautz, 2006). However, this response is inconsistent as other studies do not report improvements on DMI in ruminants (Raeth-knight et al., 2007; Sun et al., 2012), but reported a positive effect on feed efficiency. Not observing an increase in DMI, but an increase in output means that animals utilize more nutrients from the amount of DM that they consumed, therefore increasing productive efficiency. This is supported by the reports of Nocek et al. (2002), Qiao et al. (2010), and AlZahal et al. (2014), who indicate that feeding DFM increases nutrient digestibility.

Direct-fed microbials are reported to increase milk yield in dairy cows with no effects on milk composition; it is common to observe that responses are variable over

time. Noeck and Kautz (2006) observed an increase of 2.3 kg/d in milk yield for cows supplemented with a combination consisting of Enterocccus faceium, Lactobacillus plantarum, and Sacchormyces cerevisiae. Peng et al. (2012) reported an even greater increase in milk yield of 3.1 and 3.2 kg/d with Bacillus subtilis natto; interestingly the increase in milk yield was not observed until the last four weeks of the 9-wk experiment. A different study that fed Enterocccus faceium (Nocek et al., 2003), indicate an overall increase in milk yield and milk protein, whereas Luan et al. (2015) reported increases in milk yield, milk protein, and fat corrected milk (FCM) only during the second week of the experiment with Bacillus pumilus. Oetzel et al. (2007) observed increased milk fat concentration for primiparous cows and an increase in percentage of milk protein for the second lactation cows using DFM of Enteroccous faecium plus Saccharomyces cerevisiae yeast. Qio et al. (2010) only observed an increase in milk yield and milk protein for cows supplemented with Bacillus licheniformis, but not for Bacillus subtilis.

As stated before, several factors influence how animals respond to DFM supplementation; this is a persistent complication that leads to conflicting results. For example, Raeth-Knight et al. (2007), Chiquette et al. (2008), AlZahal et al. (2014) observed no differences in DMI of dairy cows supplemented with a DFM. Although, Qiao et al. (2010) observed that one DFM treatment had an increase in DMI the other DFM treatment did not influence DMI or digestibility. Raeth-Knight et al. (2007), AlZahal et al. (2014) report no differences in digestibility of neutral detergent fiber (NDF), and Raeth-Knight et al. (2007) also reported no differences on protein or starch digestibility compared to the control diets. Ferraretto and Shaver (2015) observed a trend for

decrease in DMI when supplementing a probiotic and there were several weeks that a significantly lower DMI was detected; nonetheless, feed conversion remained unaffected by treatment.

AlZahal et al. (2014) and Ferraretto and Shaver (2015) noted that milk yield, milk fat and protein for cows supplemented with a DFM were similar to the control diets. While some studies had no differences in milk yield, they did report a decrease in milk fat (Nocek and Kautz, 2006) or both fat and protein (Vieira et al., 2014).

Effects of Direct-fed Microbials in the Transition Period

The transition period for lactating dairy encompasses three weeks before calving and the subsequent three weeks into lactation (Grummer, 1995). Lactating dairy cows are in a negative energy balance during early lactation because the energy output in milk is greater than energy intake from feed; therefore, cows mobilize fat reserves to meet their energy requirements.

Nocek et al. (2003) analyzed blood glucose and insulin levels postpartum and reported that the concentrations of these analytes were higher for cows fed Enterococcus faecium. Having higher concentration of circulating glucose could indicate that more energy is available. They also noticed that concentration of non-esterified fatty acids (NEFA) was lower in cows that were supplemented with DFM. This is in accordance with another study (Peng et al., 2012) that reported having lower plasma NEFA after calving with cows that were fed Bacillus subtilis natto. Lower concentration of NEFA indicates that cows are mobilizing less energy from adipose deposits to meet

their high energy requirements. Nocek and Kautz (2006) report that cows consuming Enterococcus faecium had lower concentration of β-hydroxybutyrate postpartum. Luan et al. (2015) observed less subclinical ketosis after calving with cows consuming Bacillus pumilus as a DFM. Less ketones and ketosis suggest that supplementing probiotics can decrease the amount of energy cows take from adipose tissue. When blood glucose is made available and cows are mobilizing less fatty acids from adipose tissues, glucose can route to the mammary gland to produce more milk. These results show that DFM have potential to make the diet more energetically favorable for cows during the transition period.

Utilization of *Pediococcus* in the Dairy Industry

Pediococcus is a homofermentative, facultative anaerobe that metabolizes carbohydrates and produces lactate by degrading hexose via glycolysis (Kandler, 1983). It is a gram-positive LAB, widely used as an inoculant in silages commonly fed to dairy cows (Silva et al., 2016). To the best of our knowledge, there are no studies that have fed *Pediococcus acidilactici* as a DFM for ruminants; therefore, this section will focus on the utilization of this bacterium as a forage inoculant. It is important to highlight that one study suggests that *Pediococcus acidilactici* survives in the rumen and passes in the feces of cattle (Rodriguez-Palacios et al., 2009). From previous experiments, it is not possible to discern whether the responses observed are a result of feeding higher quality forages or if the inoculant may have had a DFM-like additive effect.

Fitzsimons et al. (1992) observed *Pediococcus acidilactic* accelerated rates of lactic acid on production and lowers the pH in silages. Cleale et al. (1990) fed a silage inoculant containing *Pediococcus acidilactici* to look at the effects on growing heifers. They report that body weights (**BW**) and DMI were greater for heifers fed the inoculant than the group consuming non-inoculated silage. They also detected an increase in digestibility for organic matter (**OM**), protein, and acid detergent fiber (**ADF**). Another study (Jatkauskas and Vrotniakienė, 2007) used the same *Pediococcus* species, but with a different combination with the addition of a different bacteria species for the inoculant, to determine the effects on rumen metabolism of dairy cows. There were no effects on rumen pH or total VFA concentration; however, there was a lower ratio of acetate to propionate for the inoculated group. Rumen protein synthesis proved to be better in cows with the inoculated silage associated with lower ammonia concentration.

Pediococcus acidilactici was used in a study on weaning piglets and positively influenced body weight and post weaning average daily gain (Di Gianocamillo et al., 2008). Broiler chickens also had a positive effect on immune response in resistance to coccidiosis when adding *Pediococcus acidilactici* to their diet (Lee et al., 2007). Lessard et al. (2009) observed that the addition of *Pediococcus acidilactici* in pigs infected with *Escherichia coli* had greater T cells and had reduced bacterial translocation to mesenteric lymph nodes.

Utilization of *Bacillus* in the Dairy Industry

Bacillus are aerobic, endospore-forming bacteria that can provide sources of amylase and protease (Kunst et al., 1997). After a halt in growth, it can reinitiate activity when growing conditions become favorable. These responses include motility, chemotaxis, and production of protein and carbohydrate hydrolyzing enzymes. *Bacillus subtilis* uses carbohydrates through the Embden-Meyerhof-Parnas glycolytic pathway coupled to the TCA cycle. It can also grow anaerobically if nitrate is present as an electron acceptor.

Bacteria in the *Bacillus* genus are cellulolytic bacteria described as an acceptable DFM due to their storage convenience and effectiveness (Seo et al., 2010). Spore forming bacteria also have greater resistance to intestinal environmental conditions (Hong et al., 2005). In addition, *Bacillus* is a gram-positive bacteria, known for its antimicrobial compound synthesis and broad enzymatic capabilities (Mongkolthanaruk, 2012). Positive effects of *Bacillus* species have been reported as increased milk yield, fat-corrected milk, and milk protein (Kritas et al., 2006, Qiao et al., 2010). Feeding efficiency for lactating dairy cows has also been increased by supplementing *Bacillus* (Ferguson et al., 2010) and altering rumen fermentation towards a higher propionate concentration (Peng et al., 2012). Specifically, *Bacillus subtilis* has shown to play some role in controlling infectious disease and improving animal productive performance in ruminants (Sun et al., 2010, Novak et al., 2012). Qiao et al. (2010) reports that while *Bacillus licheniformis* increased milk production, milk protein, and apparent digestibility, they also found that *Bacillus subtilis* did not have an increase in these variables.

Potential of *Pediococcus acidilactici* and *Bacillus subtilis* as Direct-fed Microbials for Dairy Cows

Pediococcus acidilactici being a LAB allows for a potential positive influence on balancing rumen function by sustaining a metabolically active LUB population, which is believed to prevent lactate accumulation. This ultimately can benefit the cow to increase DMI or milk production. *Bacillus* species could potentially be a suitable DFM competitor for ruminant animals because of the ability to positively impact the immune system. *Bacillus subtilis* is effective in producing antimicrobial compounds and improving immune responses during stressful periods. *Bacillus subtilis* has been used in several pig and poultry studies to successfully stimulate a positive immune response that might make a beneficial probiotic for ruminants (FAO, 2016). Improving immunity in lactating cows allows for her to distribute energy consumption necessary for milk production.

Conclusion

There are still questionable results about the effects of DFM on lactation performance in dairy cows. Some of the research suggest that the results of adding a DFM to the diet are somewhat variable with the studies reporting effects as: some positive, some with no changes, and a few negative. These conflicting results suggest further research is necessary to obtain consistent results and develop a better understanding of what conditions are more favorable to determine the efficacy of DFM. The various species and strains used as DFM make it hard to identify which ones truly

elicit a response. All of species mentioned in this manuscript have positive attributes that can make them beneficial for supplementation in ruminant diets. The uncertainty of how each one will work under different animal conditions remains a constant challenge.

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CHAPTER 2. EFFECTS OF DIET SUPPLEMENTATION WITH PROBIOTICS *Pediococcus aciliactici* OR *Bacillus subitlis* ON MILK PRODUCTION, DIGESTIBILITY, AND RUMEN FUNTION IN DAIRY COWS

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Introduction

The World Health Organization (WHO, 2002) defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". In the animal industry, probiotics are also known as direct-fed microbials (**DFM**) that can improve the gut microflora. Most DFM used in diets for ruminants consist of lactic acid-producing bacteria (**LAB**), lactic acid-utilizing bacteria (**LUB**), with some yeast products containing *Saccharomyces* and *Aspergillus* (Seo et al., 2010). In addition to DFM, substrates such as mannan oligosaccharides are called prebiotics and their function is to facilitate specific adaptations in composition and activity of the gut microflora (Slavin, 2013) to increase production in livestock (Franklin et al., 2005).

Studies demonstrate the variability in responses when supplementing DFM to dairy cows. Even though there are conflicting studies, several reports have demonstrated that supplementing DFM to dairy cows may increase milk yield, milk composition, DMI, digestibility, as well as improvements in rumen pH (Nocek et al., 2002, Nocek and Kautz, 2006, Qiao et al., 2010). These studies included a combination

consisting of *Enterocccus faceium, Lactobacillus plantarum,* and *Sacchormyces cerevisiae* and *Bacillus licheniformis*, which are commonly used in other farm animal species. Some DFM are reported to positively stimulate the innate and adaptive immune system as seen in the experiment Novak et al. (2012), where calves drenched with a *Bacillus*-based electrolyte enhanced clearance of pathogens. The combination of *Enteroccocus faceium* and yeast is a commercially available product (Probios ® Chr. Hansen) and some studies indicate that it can improve DMI, milk yield, and milk composition (Nocek et al., 2003, Nocek and Kautz, 2006). There are still novel species and strains of DFM with limited research that have potential to improve animal performance in the dairy industry, such is the case for *Pediococcus acililactici* and *Bacillus subtilis.*

Pediococcus acidilactici is a gram-positive LAB that is used in silage inoculants to increase lactic acid production and lower the pH (Silva et al., 2016). Even though there are no studies documenting the use of *P. acidilactici* as a DFM for dairy cows, the effects seen with inoculated silage may be, in part, due to the activity of *P. acidilactici* on the host animal. Cleale et al. (1990) compared inoculated silage to non-inoculated silage and determined that heifers fed the inoculated forage had greater BW, DMI, and digestibility. Similarly, Jatkauskas and Vrotniakienė (2007) worked with dairy cows being fed grass silage inoculated with *P. acidilactici* and reported increased microbial protein synthesis without affecting rumen VFA concentration or pH in dairy cows. The *Bacillus* genus include spore forming bacteria that have either mechanisms to inhibit gastrointestinal infection or by producing an antimicrobial substance that kills undesired microorganisms or suppresses their growth (Seo et al., 2010). *Bacillus subtilis* is a gram-

positive bacterium that has potential for producing endospores to help improve productive performances and positive effects on immune response in ruminants (Sun et al., 2012). Qiao et al. (2010) fed *Bacillus* as a DFM for dairy cows and reported an increase in milk yield and milk protein concentration. Kritas et al. (2006) used *Bacillus subtilis* and observed an increase in milk yield and reduced mortality in lambs.

When feeding DFM with LAB to dairy cows, the production of lactate could be maintained at a low, steady rate as opposed to rapid spikes with increased risk for acidosis (Nocek et al., 2002) when feeding readily fermentable diets. When feeding inoculated forages with *P. acidilactici* it is not clear whether the positive responses are due to the improved composition of the silage or an associative effect due to the presence of *P. acidilactici*. Therefore, we hypothesized that these DFM would have a positive effect on dairy cow performance. To the best of our knowledge, this is the first documented experiment feeding *P. acidilactici* as a DFM for dairy cows. The objectives of this experiment were to evaluate and determine the effects of *P. acidilactici* and *B. subtilis* as novel probiotics on lactation performance and, nutrient digestibility, volatile fatty acid concentration and immune response in dairy cows.

Materials and Methods

Animal Care, Housing, and Feeding

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University. Cows were housed in a free-stall barn with

individual feeding gates (Calan Broadbent Feeding System, American Calan, Northwood, NH) and fans for heat abatement. The experiment lasted for 105 d where daily care involved milking at 0700, 1500, and 2300 h, individual feeding at 0700 and 1600 h for approximately 110% of ad libitum consumption. Orts were collected, weighed, and recorded for each cow every day prior to the first feeding.

Animals, Experimental Design, and Treatments

Forty-eight multiparous Holstein cows averaging (121 ± 22 DIM) were used in a randomized complete block design. Cows were blocked by previous lactation 305 ME milk yield; two blocks contained a total of eight cows that were ruminanlly-cannulated. Test products were *Pediococcus acidilactici* 19839 and *Bacillus subtilis* 15541 (Chr-Hansen Animal Health and Nutrition, Hørsholm, Denmark). Cows were randomly assigned to 1 of 4 treatments within each block; all treatments consisted of a basal TMR (Table 1) with different top-dressed supplements: 1) control (**CON**) with no probiotics; 2) *Pediococcus acidilactici* (**PED**) 4 g fed at 1×10^{10} CFU/d; 3) *Bacillus subtilis* (**BAC**) 6 g fed at 1×10^{10} CFU/d and; 4) basal TMR supplemented with 14 g of the combination of *Enterococcus faecium* at 1×10^{10} CFU/d and yeast (**PRO**). Each of the supplements were added by weight to represent their CFU and mixed with ground corn to equal 100 g total for each daily dose during the morning feeding; CON diet was given a placebo of 100 g of ground corn.

Sampling and Data Collection

Feed Sampling. Samples of the basal TMR were collected weekly on two consecutive days and pooled to obtain a composite sample by week. Feed samples were placed in a forced-air oven at 65°C for 48 h to determine DM and then ground (1-mm screen; Wiley Mill, Arthur H. Thomas Co., Philadelphia, PA) and stored at room temperature. The diet was analyzed for nutrient composition by an external laboratory (Cumberland Valley Analytical Services, Waynesboro, PA). Analyses included DM (method 930.15; AOAC International, 2000), N (method 990.03; Leco FP-528 Nitrogen Combustion Analyzer, Leco Corp., St. Joseph, MI), NDF (Van Soest et al., 1991), starch (Hall, 2009), ether extract using diethyl ether as the solvent (method 2003.05; AOAC International, 2006), ash (method 942.05; AOAC International, 2000), and phosphorus by inductively coupled plasma (method 985.01; AOAC International, 2000).

***Milk Data Collection*.** Individual milk yields were recorded daily and averaged weekly for data analyses. Individual milk samples were collected weekly during each milking for two consecutive days and preserved using a pellet of 2-bromo-2-nitropropane-1,3 diol at room temperature. Milk samples were analyzed for milk fat, protein, lactose, and milk urea nitrogen (MUN) using Fourier transform infrared spectroscopy (MilkoScan FT+, FOSS Analytical, Eden Prairie, MN) by an external laboratory (Dairy Lab Services Inc., Dubuque, IA). Milk fat and protein yields were estimated using the corresponding milk weights at the time of collection.

***Fecal sampling*.** Indigestible neutral detergent fiber (INDF) was used as an internal marker to determine total tract digestibility based on fecal samples collected during the last week of the experiment. Fecal grab samples were collected every 8 hours for 2 consecutive days from all cows; the starting time for fecal sampling was shifted 4 h during the second day of collection to account for diurnal variation. Fecal samples were then pooled by cow, placed in a forced-air drying oven at 65°C for 48 h to determine DM, and then ground (1-mm screen; Wiley Mill, Arthur H. Thomas Co., Philadelphia, PA). A sub sample of TMR and pooled feces from each cow were analyzed for nutrient content that included DM (method 930.15; AOAC International, 2000), ash (method 942.05; AOAC International, 2000), NDF (Van Soest et al., 1991), and N (method 990.03; Leco FP-528 Nitrogen Combustion Analyzer, Leco Corp., St. Joseph, MI). Determination of INDF was performed in quadruplicate by incubating 5 × 10 cm dacron bags containing 1.25 g of sample material in two cannulated cows for 288 h (Huhtanen et al., 1994). The bags were then washed, dried, and analyzed for NDF (Van Soest et al., 1991) to estimate fecal output.

***Rumen Fluid Collection*.** Rumen fluid was collected from the eight cannulated cows on the last day of the experiment following a 5-d adaptation to housing in individual box-stalls. Starting at the time of feeding, samples of rumen fluid were collected every 2 h during a 24-h period. Subsamples of digesta from the rumen were collected by hand through the canula, mixed, and strained through four layers of cheesecloth to collect rumen fluid into a plastic container. Rumen pH was measured immediately after collection using a hand-held pH meter; two aliquots of rumen fluid

were stored in screw-capped 50 mL tubes and were immediately stored at -20˚C for later analyses. One of these subsamples was kept as a backup whereas the other one was used for VFA analysis. Samples were thawed and centrifuged at 5°C at 9,000 r.p.m, for 15 min; after centrifugation, 4 mL of supernatant were acidified with 0.70 mL of 25% (wt/vol) metaphosphoric acid and frozen at −20°C until being analyzed for VFA. Ruminal VFA were determined using gas chromatography (Varian CP-3800, Palo Alto, CA) with a Nukol Fused silica capillary column (15 m x 0.25 mm x 0.25 µm film thickness; Supelco Inc., Bellefonte, PA) and flame ionization detector. Samples were analyzed with a programmed temperature gradient at 143°C initial temperature for 1.5 min, then temperature increased by 15 °C/min up to 200°C, then held at this temperature for 0.5 min. The temperature of the injector and detector was 200˚C and 250°C, respectively. The carrier gas was helium, and column flow rate was 2 mL/min.

Blood Sampling and Analysis. Blood samples were collected bi-weekly at 1500 h by venipuncture of the coccygeal vein using vacuum evacuated collection tubes. Samples were immediately stored at 4˚C overnight until sent to the Clinical Pathology Laboratory of Iowa State University to be analyzed for complete blood count with automated differential.

Statistical Analyses

Data were analyzed using a generalized mixed model (SAS version 9.3, SAS Institute Inc., Cary, NC) with week, treatment and their interaction as fixed effects with pre-experiment milk yield as a covariate and cow and block as random effects. The error

term was assumed to be normally, independently, and identically distributed, with variance σ^2_e. Data obtained from ruminal fluid were analyzed as repeated measures using the first-order auto-regressive covariance structure. The effects of treatment, hour and treatment × hour interaction were considered as fixed and cow was considered as a random effect. Statistical significance for all treatments effects was declared at $P \leq 0.05$; trends are discussed at $P \leq 0.15$. All mean results are presented as least squares means ± the largest standard error of the mean unless stated otherwise.

Results

Direct-fed microbials are used in dairy rations to support or improve performance, this study investigated the effects of *Pediococcus acidilactici* 19839 and *Bacillus subtilis* 15541, on lactation performance, digestibility, rumen pH, and VFA concentration in lactating dairy cows. Lactation performance is presented in Table 2; DMI was similar ($P = 0.20$) across all treatments with an average of 24.3 ± 0.75 kg/d. Although no statistical significance was observed, the weekly pattern of DMI (Figure 1) depicts that cows consuming PED consumed more DM, particularly during the first eight weeks of the experiment. Overall, milk yield was similar ($P = 0.82$) at the end of the 15-wk period (Figure 2) with an average 37.4 ± 1.41 kg/d. Data were also analyzed in three 5-week periods and these results showed that feeding PED led to a greater ($P < 0.05$) milk yield for the first 5-wk periods by 3.9 ± 2.87 kg/d, respectively, compared to the average of all other treatments. Milk composition was similar for fat ($P = 0.93$) and protein ($P = 0.71$) across all treatments, averaging 3.63 ± 0.16% and 3.05 ± 0.06%, respectively. Similarities between treatments were observed for milk urea nitrogen ($P =$

0.33) and energy correct milk (P = 0.61) with averages of 12.6 ± 0.39 mg/dL and 36.8 ± 1.52 kg/d. Feed efficiency corresponds with this pattern with an average of 1.53 ± 0.06 across treatments (P = 0.80).

We observed similar apparent digestibility of DM (P = 0.38; Table 3) and organic matter (P = 0.44) across treatments with averages of 66.65 ± 1.48 % and 68.88 ± 1.43 %, respectively. Digestibility of NDF and ADF were similar across treatments with an average of 50.07 ± 2.23 % and 31.17 ± 3.2 %. Protein digestibility averaged 67.11 ± 1.81 % across all treatments.

Figure 3 shows the pH measurements by treatment over a 24-h period. Mean rumen pH was 5.6 ± 0.05 across treatments (P = 0.29). We observed similarities (P = 0.70; Table 4) across treatments for total VFA concentrations, the average was 145 ± 12.2 mMol/L. Concentrations of acetate, propionate, and butyrate were similar ($P \geq$ 0.18) across treatments with averages of 57.1 ± 1.84 mol/100 mol, 26.6 ± 2.26 mol/100 mol, 11.2 ± 0.68 mol/100 mol. Consequently, the ratio for acetate to propionate was also similar (P = 0.45) across treatments with an average of 2.24 ± 0.26 mol/100 mol. Valerate and isobutyrate were also similar across treatments (P = 0.31; P = 0.45) and averaged 2.04 ± 0.25 mol/100 mol and 0.59 ± 0.26 mol/100 mol. However, there was a trend for isovalerate to be greater for PED by 0.30 mol/100 mol compared to CON.

Blood data from this experiment is presented in table 5. Concentrations of white blood cells (WBC) were different among treatment (P < 0.01); there was an increase in the concentration of WBC for PED and decrease for BAC treatments being 9.97 and 8.74

± 0.20× 10^3/μL, respectively. The differences occur from the concentration of neutrophils 3.88 and 3.31 ± 0.15 × 10^3/μL, as well as the concentration of basophils with 0.09 and 0.07 ± 0.003 × 10^3/μL.

Discussion

Productive responses upon feeding bacterial DFM are variable depending on species of DFM, stage of lactation, and diet composition (Seo et at., 2010). For example, Qiao et al. (2010) reported an increase in milk production with *Bacillus licheniformis,* but no difference in milk yield with *Bacillus subtilis*. Our observations for overall similarities in milk yield between the control and experimental treatments are in accordance with other studies (AlZahal et al., 2014, Luan et al., 2015). Similar to our experiment, Luan et al. (2015) observed temporal differences in milk yield during wk 1 to 4 and wk 9 to 10 when supplementing *Bacillus pumilus* 8G-134 to dairy cows. Transient increases in milk yield were also reported by Peng et al. (2012) who observed similar milk yield throughout their 9-wk experiment and only greater milk yield during the last four weeks for cows consuming a supplement with *Bacillus subtilis* compared to control cows. Despite no statistical differences, the pattern of DMI and milk yield in our experiment suggests that supplementing with *P. acidilactici* may modulate milk yield by promoting greater DMI. This observation would be in agreement with studies by Nocek and Kautz (2006) and Peng et al. (2012) who have reported increased milk yield and DMI when cows consume bacterial DFM. Modulation of DMI is a mode of action for improved performance with no evident advantages on nutrient digestibility. Opposite to our hypothesis of increased digestibility upon feeding DFM, we observed no differences in

apparent total tract digestibility of nutrients determined after 105 days of supplementation. Regrettably, we did not collect fecal samples to estimate digestibility on a weekly basis to evaluate a possible mechanism that could explain the transient increase in milk yield. Future experiments should consider taking more periodic sampling to obtain more information about digestibility over time.

The VFA concentrations in ruminants are variable based on the mode of action of each type of DFM. Raeth-Knight et al. (2007) supplemented a combination of *Lactobacillus acidophilu*s and *Propionibacteria freudenreichii,* and reported no changes in rumen pH or total VFA concentrations. This is in accordance with our observations with similar total VFA concentrations. Consequently, rumen pH was also similar across treatments. Nocek et al. (2002) reported no differences in average pH with feeding LAB DFM, but did see changes between the different levels of supplementation and successfully stimulated LUB, suggesting that the lactate produced by the DFM induces changes in population of lactate utilizing bacteria. In contrast with our study, (Philippeau et al., 2017) the combination of supplementation of *Propionibacterium* P63 with *Lactobacillus plantarum* or *Lactobacillus rhamnosus* increased ruminal pH compared to a no DFM, but this still had no effect on VFA concentration.

Immune response from supplementing DFM have reported in swine and poultry studies (Lessard et al., 2007; Lee et al., 2007). The calf study from Novak et al. (2012) utilized an electrolyte drench with *Bacillus,* the authors reported improved immunity during scouring and after by clearing the pathogenic infection from the GIT. There is limited research focusing on evaluation of DFM on the immune system of lactating dairy

cows; we observed an increase of WBC with *P. acidilactici* and a decrease in WBC with *B. subtilis*. We believe that this increase in WBC is the result of stimulating the immune system. Although speculative, our observation on immune-modulation may have positive implications when cows are immunosuppressed or undergoing a phase of stress. Additional research inducing an immune challenge is warranted to validate this theory.

Conclusion

The inclusion *Pediococcus acidilactici* and *Bacillus subtilis* maintained milk composition with no increase in milk yield. Supplementation with *B. subitlis* did not result in notable performance advantages. However, we observed that cows supplemented with *P. acidilactici* had numerically greater DMI and milk yield during the first five weeks of supplementation possibly due to increased dry matter intake. Despite feeding lactic acid producing bacteria, rumen fermentation was stable and no detrimental effects were observed on fiber digestibility. In addition, supplementing *P. acidilactici* DFM resulted in the greatest concentration of white blood cells; this suggests an immuno-stimulatory effect. Even though there is evidence of the potential positive role of these novel DFM tested in this experiment, further research is warranted to fully elucidate the mode of action.

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Tables and Figures

Table 1. Ingredients and analyzed chemical composition of the basal diet (n = 15, average ± SD)

Item	Value
Dietary ingredient (% of DM)	
Corn Silage	33.7
Wet Corn Gluten Feed	19.9
Alfalfa Hay	15.5
Grain mix[1]	30.9
Chemical (% of DM, except for DM)	
DM	50.4 (2.3)
CP	16.0 (1.2)
NDF	35.0 (3.1)
Lignin	3.91 (0.51)
Starch	20.6 (2.0)
EE	4.7 (0.4)
NFC[2]	38.6 (2.3)
Ash	7.3 (0.4)
Ca	0.71 (0.17)
P	0.51 (0.05)
Mg	0.37 (0.05)
K	1.57 (0.11)
S	0.28 (0.03)
Na	0.59 (0.06)
Cl	0.51 (0.05)
Fe (mg/kg)	232 (34.9)
Mn (mg/kg)	67.8 (7.3)
Zn (mg/kg)	98.0 (7.6)
Cu (mg/kg)	23.9 (1.6)

[1]Grain Mix- contains expeller-cooked soybean meal (SoyPlus, West Central Cooperative, Ralston, IA), yeast and yeast culture media extracts (Diamond V, Diamond V Mills Inc., Cedar Rapids, IA), molasses (QLF, Dodgeville, WI).

[2]NFC = Non-fiber carbohydrates calculated by difference 100 – (%NDF + % CP + % EE + % Ash)

Table 2. Effects of dietary supplementation of two novel DFM on the performance of dairy cows.

	Dietary Treatment[1]				SEM[2]	*P* – value[3]
	CON	PRO	BAC	PED		
DMI (kg/d)	24.1	23.7	23.9	25.6	0.75	0.20
Milk yield (kg/d)	37.3	37.7	36.6	38.1	1.41	0.82
Energy corrected milk[4] (kg/d)	37.6	36.5	35.4	37.7	1.52	0.61
Feed effiency[5]	1.57	1.51	1.49	1.54	0.06	0.80
Fat (%)	3.71	3.57	3.60	3.62	0.16	0.93
Fat yield (kg/d)	1.35	1.26	1.27	1.43	0.08	0.35
Protein (%)	3.07	3.06	3.06	2.99	0.06	0.71
Protein yield (kg/d)	1.12	1.08	1.08	1.17	0.06	0.53
MUN (mg/dL)	12.8	12.0	12.7	12.8	0.39	0.33
BW (kg)	678	655	667	704	16.8	0.14
BCS[6]	2.90	2.83	2.91	2.84	0.06	0.65

[1] CON= control; Probios = *Enterococcus faecium* + yeast; *Bacillus* = *Bacillus subtilis* 15541*, Pediococcus = Pediococcus acidilactici* 19839.

[2] Highest standard error of treatment mean is shown.

[3] Main effect of treatment, the term week × treatment was not significant and was removed from the model.

[4]ECM = [milk fat (kg) × 16.216] + [milk yield (kg) × 0.4323].

[5] Feed efficiency = Energy corrected milk / dry matter intake

[6]1-5 scale (Wildman et al., 1982).

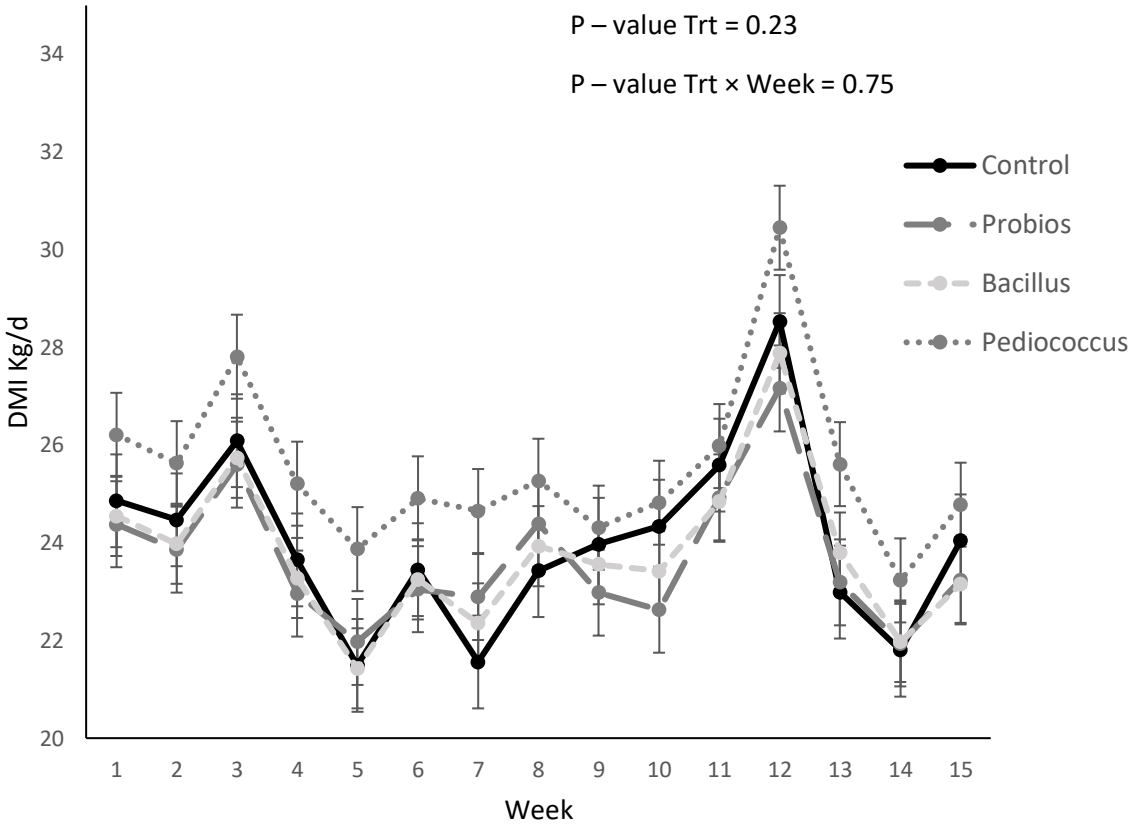

Figure 1. Supplementation with *Bacillus subtilis* 15541 and *Pediococcus acidilactici* 19839 on weekly DMI of dairy cows

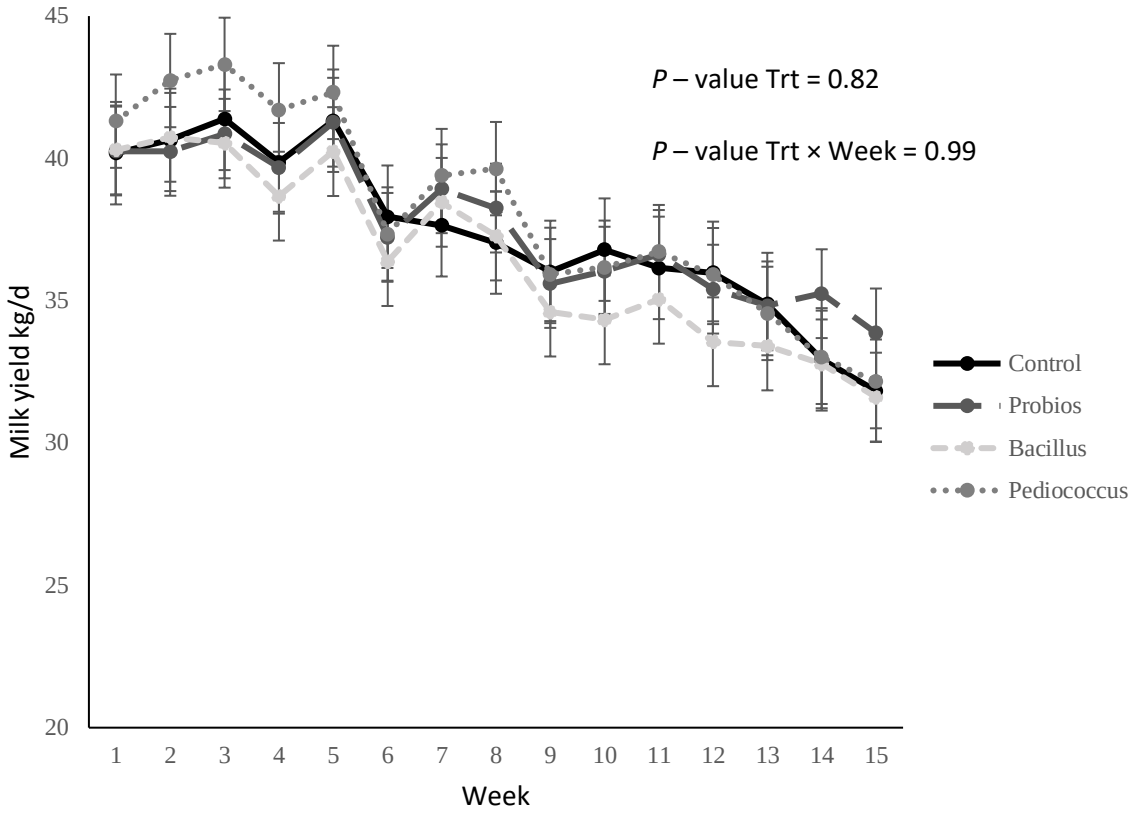

Figure 2. Supplementation with *Bacillus subtilis* 15541 and *Pediococcus acidilactici* 19839 on weekly milk yield of dairy cows

Table 3. Effects of dietary supplementation of two novel DFM on nutrient digestibility in dairy cows.

	Dietary Treatment[1]				SEM[2]	P – value[3]
	CON	PRO	BAC	PED		
Digestibility, %						
Dry Matter	68.59	65.85	66.89	65.27	1.48	0.38
Organic Matter	70.52	68.21	69.27	67.50	1.43	0.44
NDF	53.16	48.14	50.45	48.52	2.23	0.35
ADF	34.33	29.72	31.95	28.69	3.42	0.63
Crude Protein	68.00	66.72	68.00	65.74	1.81	0.73

[1] CON= control; Probios = *Enterococcus faecium + yeast*; Bacillus = *Bacillus subtilis 15541, Pediococcus = Pediococcus acidilactici 19839*

[2]Highest standard error of treatment mean is shown.

[3]Main effect of treatment

Table 4. Effects of dietary supplementation of two novel DFM on rumen pH and VFA concentration in dairy cows.

	Treatment[1]				SEM[2]	*P* – value	
	CON	PRO	BAC	PED		Trt	Trt × Time
Rumen pH,							
Minimum	5.35	5.29	5.34	5.21	0.05	0.26	
Mean	5.75	5.75	5.65	5.61	0.08	0.29	<0.01
Maximum	6.65	6.85	6.57	6.49	0.18	0.59	
Total VFA mMol/L	141	145	141	154	12.2	0.70	<0.01
VFA, mol/100 mol							
Acetate	57.8	58.1	55.5	57.0	1.84	0.56	<0.01
Propionate	25.3	25.5	29.4	26.1	2.26	0.36	<0.01
Butryate	11.6	11.5	10.0	11.6	0.68	0.18	<0.01
Valerate	2.25	1.73	2.15	2.02	0.25	0.31	<0.01
Isovalerate	2.41	2.54	2.36	2.71	0.10	0.07	<0.01
Isobutyrate	0.60	0.59	0.55	0.62	0.04	0.45	<0.01
Acetate:Propionate	2.54	2.30	1.91	2.19	0.26	0.45	<0.01

[1] CON= control; Probios = *Enterococcus faecium* + *yeast*; Bacillus = *Bacillus subtilis 15541, Pediococcus = Pediococcus acidilactici 19839*

[2]Highest standard error of treatment mean is shown.

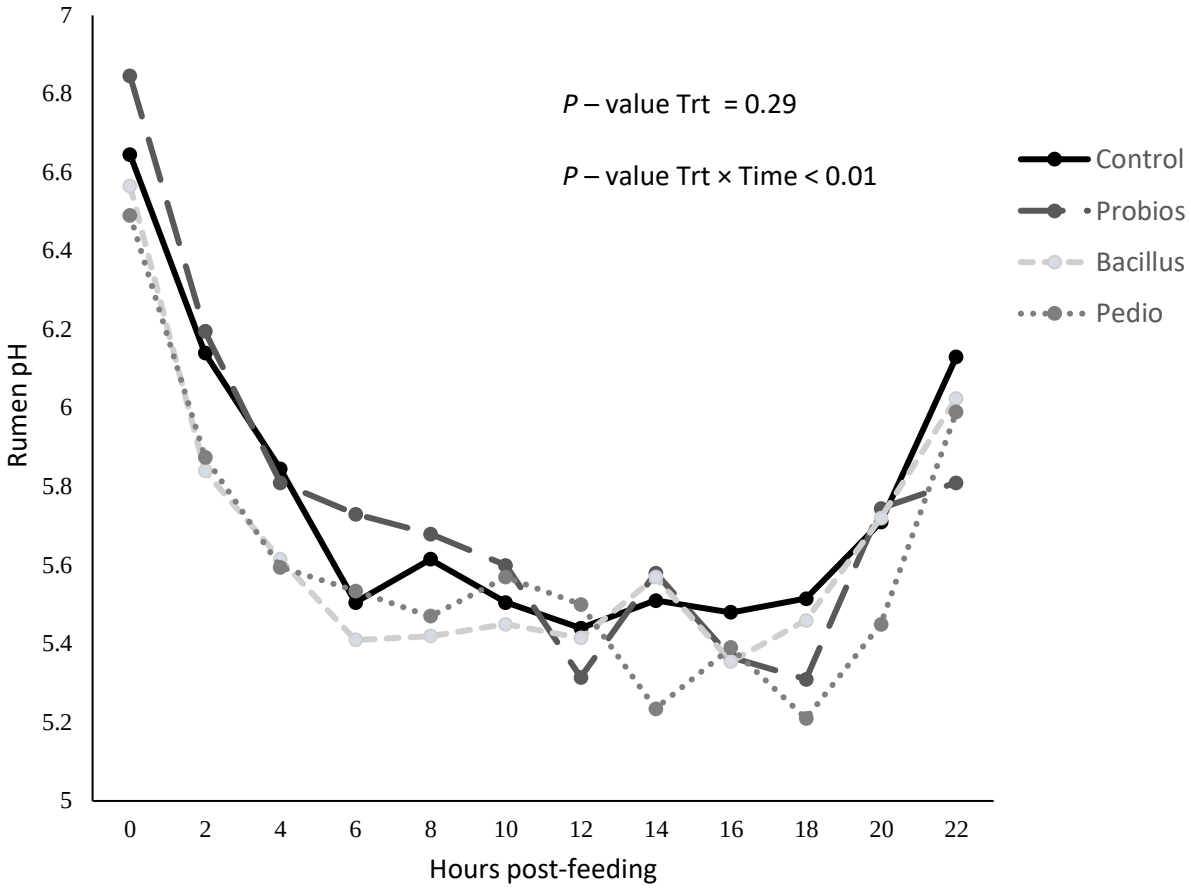

Figure 3. Supplementation with *Bacillus subtilis* 15541 and *Pediococcus acidilactici* 19839 on diurnal pattern of rumen pH in dairy cows

Table 5. Effect of dietary supplementation of two novel DFM on white blood cell count(WBC).

	Treatments[1]				SEM[2]	*P*-value[3]
	CON	PRO	BAC	PED		
WBC (× 10^{3}/µL)	9.52^{ab}	9.36^{b}	8.74^{c}	9.97^{a}	0.20	<0.01
Neutrophils (× 10^{3}/µL)	3.96^{a}	3.67^{ab}	3.31^{b}	3.88^{a}	0.15	<0.01
Lymphocytes (× 10^{3}/µL)	4.67	4.83	4.57	5.24	0.28	0.56
Monocytes (× 10^{3}/µL)	0.43	0.40	0.36	0.43	0.02	0.30
Eosinophils (× 10^{3}/µL)	0.36	0.34	0.40	0.28	0.28	0.98
Basophils (× 10^{3}/µL)	0.07^{b}	0.09^{a}	0.07^{b}	0.09^{a}	0.003	<0.01

[1] CON= control; Probios = *Enterococcus faecium* + *yeast*; Bacillus = *Bacillus subtilis 15541, Pediococcus = Pediococcus acidilactici 19839*

[2]Highest standard error of treatment mean is shown.

[3]Main effect of treatment